EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure for Test Microbes for the AOAC Use-Dilution Method, AOAC Germicidal Spray Products Test, Germicidal Towelette Products Test, AOAC Confirmatory Tuberculocidal Test, and the AOAC Sporicidal Activity Test Method: Culture Initiation, Culture Maintenance and Quality Control

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1.0 SCOPE AND APPLICATION:

1.1 This protocol describes the procedures used to ensure the purity and integrity of the test microbes used by the OPP Microbiology Laboratory.

2.0 DEFINITIONS:

2.1 API = Analytical Profile Index

2.2 DI = De-ionized Water

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in SOP MB-01, Lab Biosafety.

4.0 CAUTIONS:

4.1 Use aseptic techniques to prevent contamination.

5.0 INTERFERENCES: None

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable about and to comply with the laboratory's culturing and disinfectant testing procedures. Documentation of training and familiarization with these requirements can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Incubator with temperature at 37±1°C

7.2 Incubator with temperature at 30±1°C

7.3 *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Bacillus subtilis* (ATCC 19659); ordered and received directly from ATCC.

7.4 Selective media in Petri dishes: Pseudosel Agar, Mannitol Salt Agar, and Middlebrook 7H9 agar.

7.5 *Mycobacterium bovis* (BCG); ordered and received directly from Organon Teknika.

7.6 BBL Gram Stain Kit

7.7 BBL TB Quick Stain Kit

7.8 API Identification Strips, API-20 NE, API-STAPH, and API 50 CH (bioMérieux Inc., Hazelwood, Missouri)

7.9 API Reagents for identification strips (bioMérieux Inc.)

7.10 Vitek 32 System for the automated identification of microorganisms

7.11 Vitek 32 Identification Cards (GNI, GPI+, and B)

7.12 #18 Broth (Trypticase Soy Broth (BBL 11768) manufactured by Becton Dickinson Microbiological Systems; available from Fisher Scientific)

7.13 #3 Broth (Nutrient Broth (DIFCO 0003) manufactured by Becton Dickinson Microbiological Systems; available fom Fisher Scientific)

8.0 INSTRUMENT OR METHOD CALIBRATION: Not applicable

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Refer to section 10.0 for storage conditions for test microbes.

10.0 PROCEDURE AND ANALYSIS:

10.1 *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Bacillus subtilis*

10.1.1 Culture Initiation.

10.1.1.1 Annually obtain lyophilized cultures of *Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538), and *Bacillus subtilis* (ATCC 19659) from the American Type Culture Collection (ATCC).

10.1.1.2 Initiate cultures per ATCC recommendations from the Propagation Procedure section of the Product Information Sheet that accompanies each organism as outlined below.

10.1.1.3 Open vial of freeze dried organism per instructions.

10.1.1.4 For *Staphylococcus aureus* and *Pseudomonas aeruginosa*, using a single tube (5-6 mL) of #18 Broth (Trypticase Soy Broth - BBL 11768) withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet. For *Bacillus subtilis*, using a single tube (5-6 mL) of #3 Broth (Nutrient Broth - DIFCO 0003) withdraw approximately 0.5 to 1.0 mL with a Pasteur or 1.0 mL pipette and rehydrate the pellet.

10.1.1.5 Using several drops of the suspension, inoculate a second tube of broth (#18 Broth for *Staphylococcus aureus* and *Pseudomonas aeruginosa* and #3 Broth for *Bacillus subtilis*).

10.1.1.6 Incubate broth culture at 37±1°C for 24±2 hours for *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Incubate broth culture at 30±1°C for 24±2 hours for *Bacillus subtilis*.

10.1.2 Culture Identification and Quality Control.

10.1.2.1 Initial identification confirmation testing for quality control (QC) and the generation of the first series of stock cultures will be performed concurrently using the 24±2 hours old broth cultures from step 10.1.1.5.

10.1.2.2 Following the incubation period, streak a loopful of each organism (an isolation streak)

onto a plate of trypticase soy agar (TSA). For *S. aureus* and *P. aeruginosa*, also streak a loopful onto a plate of the appropriate selective media (mannitol salt media for *S. aureus,* pseudosel media for *P. aeruginosa*). Selective media is not used for *Bacillus subtilis*.

10.1.2.3 Incubate plates at 37±1°C for 24±2 hours.

10.1.2.4 Following the incubation period, record the colony morphology on the TSA and selective media plates. See Table 1 for details on cell and colony morphology, and colony characteristics on selective media.

10.1.2.5 For *S. aureus* and *P. aeruginosa*, note the organism's growth characteristics on selective media (colony size, color, texture, etc.) and check for consistency with the genus and species of the organism to be tested (round, shiny, and yellow for *S. aureus,* and flat, greenish-yellow, and opaque for *P. aeruginosa*).

10.1.2.6 For each organism, perform a Gram stain from growth taken from the TSA plate. Perform the Gram stain according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000 × magnification (oil immersion).

10.1.2.7 Vitek 32 System: Using growth from an 18-24 hour TSA plate, perform the necessary tests for each organism as outlined in the PINSERT printout available from the Vitek 32 System and the instructions stated in the Vitek manual (see SOP QC-16, VITEK: Culture Identification Numbers).

10.1.2.8 API Assays: Alternatively, using growth from a

24±2 hour TSA plate, perform an API assay. To assay for *S. aureus,* use an API Staph test strip, following the API manufacturer's instructions. For *P. aeruginosa,* use an API 20 NE test strip, following the API manufacturer's instructions. For *B. subtilis*, use the API 50 CH test strip.

10.1.2.9 As soon as the API profile numbers are generated, consult the API reference manual to determine the identity of the organism (see ref. 15.2 and 15.3).

10.1.2.10 Gram stain reactions and colonial morphology of the test microbes can be found in Table 1 in section 10.3.

10.1.2.11 Record all confirmation results on the Test Microbe Confirmation Sheet (see 16.3).

10.1.3 Generation of Stock Cultures.

10.1.3.1 Use the 24±2 hour broth cultures discussed in 10.1.1.6 to initiate stock cultures.

10.1.3.2 For *S. aureus* and *B. subtilis,* streak inoculate six nutrient agar slopes. For *P. aeruginosa*, stab six cystine trypticase agar (CTA) tubes.

10.1.3.3 Incubate the *S. aureus* slopes and *P. aeruginosa* stabs at 37±1°C for 48±2 hours. Incubate the *B. subtilis* slopes at 37±1°C for 24±2 hours.

10.1.3.4 Following incubation, store the cultures at 2-5°C for 30 days. These cultures are identified as the "stock cultures". Begin stock culture transfers (outlined in section 10.1.5 under Culture Maintenance) on the 28th day of the 30 day storage period.

10.1.3.5 From a set of six stock cultures, one is used every 30 days for QC and to generate new stock cultures, four may be used per month (one/week) for generation of test cultures, (see SOP MB-05, Use Dilution Method; SOP MB-06, Testing Spray Disinfectants; and SOP MB-09, Testing Towlette Disinfectants) and one is a back-up tube.

10.1.4 Monthly QC of Stock Cultures.

10.1.4.1 Monthly QC of stock cultures may occur just prior to or concurrently with stock culture transfers. Use one refrigerated stock culture tube and streak a loopful on a plate of TSA and a plate of the appropriate selective media noted in section 10.1.2.2.

10.1.4.2 Incubate the plates at 37±1°C for 24±2 hours (18-24 hours for use in the Vitek 32 System). Follow steps outlined in section 10.1.2 to confirm the identity of the organism.

10.1.5 Culture Maintenance.

10.1.5.1 On the 28th day of the 30 day stock culture storage period, initiate stock culture transfers. Use the same refrigerated stock culture tube used for Monthly QC described in 10.1.4.1 to inoculate 6 new stock cultures tubes as outlined in 10.1.3.2.

10.1.5.2 Incubate the new stock cultures as indicated in 10.1.3.3.

10.1.5.3 Following the incubation period, store the stock cultures at 2-5 °C for 30 days.

10.2 *Mycobacterium bovis* (BCG)

10.2.1 Culture Initiation.

10.2.1.1 Obtain lyophilized cultures of *M. bovis* (BCG) from Organon Teknika.

10.2.1.2 Reconstitute the lyophilized culture with 1 mL of sterile DI water. Inoculate two Middlebrook 7H9 (M7H9) agar plates with approximately 0.1 mL of the rehydrated culture. Spread the inoculum with a sterile glass hockey stick.

10.2.1.3 Add 0.2 mL of the remaining rehydrated culture to each of 4 tubes of Modified Proskauer Beck broth (MPB).

10.2.1.4 Incubate the M7H9 agar plates and MPB broth tubes for 15 to 20 days at 37±1°C or until there is sufficient growth.

10.2.2 Culture Identification and Confirmation.

10.2.2.1 Note the organisms' growth characteristics on the M7H9 media (colony size, color, texture, etc.). Check for consistency with the genus and species of the organism (off-white to buff-colored, raised, and rough for *M. bovis* (BCG)) (see Table 10.3). *M. bovis* (BCG) is a slow growing organism. Colonies become visible on M7H9 agar in approximately 14 days.

10.2.2.2 Following the incubation period, perform an acid fast stain on a smear of the *M. bovis* (BCG) culture as stated in the manufacturer's instructions. The smear should be prepared from the MPB broth tube or from a M7H9 plate and observed using brightfield microscopy at 1000× magnification.

10.2.2.3 A description of the acid fast stain reaction and the colony morphology of *M. bovis* (BCG) can

be found in Table 1 in section 10.3.

10.2.2.4 Record all confirmation results on the Test Microbe Confirmation Sheet (see 16.3).

10.2.3 Generation of Stock Cultures and Stock Culture Maintenance.

10.2.3.1 Once the confirmation steps are completed and appropriate results are obtained for *M. bovis* (BCG), use the 15 to 20 day old MPB broth cultures to initiate stock cultures.

10.2.3.2 Streak 20-24 M7H9 agar slopes using the 1-4 tubes of MPB broth cultures of *M. bovis* (BCG).

10.2.3.3 Up to sixteen of the 20-24 stock slopes will be used to generate cultures for testing each month (see SOP MB-06, Testing Spray Disinfectants; SOP MB-07, Confirmatory Tuberculocidal Method; and SOP MB-09, Testing Towlette Disinfectants), four will be stored as extras, and 2-4 will be used to initiate the next month's stock culture of 20-24 M7H9 tubes.

10.2.3.4 Incubate the new stock transfers for 15 to 20 days at 37±1°C. Store at 2-5°C

10.2.4 QC of Stock Cultures.

10.2.4.1 Each month or 6 weeks, for quality control purposes, select one of the 2-4 stock slopes used to generate the additional 20-24 M7H9 slopes and streak a loopful of growth onto a plate of M7H9 agar.

10.2.4.2 Incubate the plate for 21-25 days at 37±1°C. Evaluate the colony morphology and perform

an acid fast stain as described in sections 10.2.2.2 through 10.2.2.4.

10.2.4.3 Acid fast stain reaction and colony morphology of *M. bovis* (BCG) can be found in Table 1 in section 10.3. Record observations on the Test Microbe Confirmation Sheet (16.3).

10.2.5 Culture Maintenance.

10.2.5.1 Each month or 6 weeks, use 2-4 stock slopes to generate an additional 20-24 M7H9 slopes. Inoculate the fresh M7H9 slopes by transferring a loopful of *M. bovis* (BCG) growth from an established tube to each of the 20-24 tubes.

10.2.5.2 Incubate the stock culture slopes at 37±1°C for 15 to 20 days. Store at 2-5°C.

10.3 Table 1. Typical Growth Characteristics of *P. aeruginosa, S. aureus, B. subtilis,* and *M. bovis* (BCG) (see ref. 15.4, 15.5 and 15.6).

	*P. aeruginosa**	*S. aureus**	*M. bovis* (BCG)**	*B. subtilis**
Gram rxn.	negative	positive	positive	positive
Acid Fast rxn.	N/A	N/A	Acid Fast	N/A
Typical Growth Characteristics on Solid Media				
Mannitol Salt	N/A	circular, small, yellow colonies, agar turning fluorescent yellow	N/A	N/A
Pseudosel	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	N/A	N/A	N/A
Middlebrook 7H9	N/A	N/A	rough, raised, thick colonies with a nodular or wrinkled surface and an irregular thin margin, off-white to faint buff, or even yellow	N/A
TSA	flat, opaque to off-white, round spreading	small, circular, yellow glistening	N/A	opaque, rough, dull, round, low convex colonies with irregular margins
Typical Microscopic Characteristics				
Cell dimensions	0.5-1.0 μm in diameter by 1.5-5.0 μm in length*	0.5-1.5 μm in diameter*	0.3-0.6 μm in diameter by 1-4 μm in length**	0.8-1.0 μm in diameter by 3.5-5.0 μm in length*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters	rods, straight or slightly curved, occurring singly and in occasional threads	rods, singly or in pairs, motile by peritrichous flagella, production of central spores

*After 24±2 hours
**After 15-20 days

10.4 Supply Control Number

10.4.1 All cultures are given a supply control number upon receipt (see SOP QC-09, Control Numbers).

10.4.2 The supply control number will consist of the date received (R) and the date the ampule expires (E).

10.4.2.1 For example, a *S. aureus* dehydrated ampule is received on 06-21-00 and expires on 10-07-01. The supply control number would be R062100-E100701. If a *M. bovis* (BCG) dehydrated ampule is received on 07-24-00 and expires on 05-04-01, the supply control number would be R072400E050401.

10.5 Microbe Received and Microbe Expiration Number (MRME).

10.5.1 The MRME number will consist of the date received (MR) and the date the reconstituted microbe expires (ME). The ME number is not required for *M. bovis* (BCG) because the culture does not expire.

10.5.1.1 For *P. aeruginosa*, *S. aureus*, and *B subtilis*. A culture received on 06-21-00 and reconstituted on 07-28-00 would receive a culture notation of MR062100ME072801, where "MR" represents microbe received and "ME" represents the date the reconstituted microbe expires.

10.5.1.2 Once reconstituted, *P. aeruginosa*, *S. aureus*, and *B. subtilis* can only be transferred for a period of one year.

10.5.1.3 Once expired, the cultures must be autoclaved and discarded and a new culture initiated from a new lyophilized ATTC lot.

10.5.1.4 For *M. bovis* (BCG). *M. bovis* (BCG) is not

required to be replaced annually. Therefore, the culture notation will only consist of the MR number.

10.5.1.5 Continuous transfers of *M. bovis* (BCG) may be made unless the organism has been compromised.

10.5.2 Additional Culture Notation. The MRME culture notation will have a suffix as follows: for *Staphylococcus* the suffix will be S; for *Pseudomonas* the suffix will be P; for *Bacillus* the suffix will be B; for *Mycobacterium* the suffix will be M.

10.5.2.1 Thus, the final culture notation after reconstitution for *Staphylococcus*, for example, would be MRXXXXXXMEXXXXXX-S. The culture notation for *M. bovis* (BCG) would be MRXXXXXX-M.

10.7 Culture Transfer Notation of Test Microbes.

10.7.1 See footnotes for Organism Culture Tracking Form for transfer notations (see 16.1). For example, *Staphylococcus* test culture notation would be MRXXXXXXMEXXXXXX-S-06-04TC, where 06 is the monthly transfer (from the stock culture tube) and 04 is the 4th daily transfer; TC is applied to indicate a test culture.

10.7.2 See footnotes for Organism Culture Tracking Form for *M. bovis* (BCG) transfer notations (see 16.2). For *Mycobacterium*, test culture notation would be MRXXXXXX-M-11-03, where 11 represents the month of transfer (November) and 03 represents the 3rd week of the month for that transfer. TC is applied to identify the test culture. Since test culture transfers typically occur every Monday, the weeks of each month are numbered consecutively starting with the 1st Monday of the month (as 01) and ending with the last Monday of the month (depending on the # of Mondays in the month, as either 04 or 05).

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the Test Microbe Conformation Sheet and the Organism Culture Tracking Form. Completed forms are archived in notebooks kept in locked file cabinets in the file room D217. Only authorized personnel have access to the locked files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate record form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 If the results of quality control do not verify the identity of the test organism, then the culture is discarded and a new culture is initiated. New stock cultures are established as outlined in section 10.0 of this SOP.

15.0 REFERENCES:

15.1 bioMérieux S.A. 1995. Industrial Vitek Reference Manual No. 510713-1, Revision Date 07-1995. bioMe′rieux Vitek, Inc., Hazelwood, MO.

15.2 bioMérieux S.A. 1997. Analytical Profile Index Reference Book Number 20 090 (20 NE), 6th Edition. Marcy-l′Etoile, France.

15.3 bioMérieux S.A. 1997. Analytical Profile Index Reference Book Number 20 590 (Staph),4th Edition. Marcy-l′Etoile, France.

15.4 Holt, J., Krieg, N., Sneath, P., Staley, J. and Williams, S. eds. 1994. Bergey's Manual of Determinative Bacteriology, 9th Edition. Williams & Wilkins, Baltimore, MD.

15.5 Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD.

15.6 Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD.

16.0 FORMS AND DATA SHEETS:

16.1 Organism Culture Tracking Form

16.2 Organism Culture Tracking Form for *Mycobacterium bovis* (BCG)

16.3 Test Microbe Confirmation Sheet

ORGANISM CULTURE TRACKING FORM

OPP Microbiology Laboratory

Organism:		Control Number:	

Date	Time	Init.	Subculture Source	Transfer*		Media Inoculated (and # inoc.)	Media Prep No.	Incubation Conditions	Comments
				Monthly	Daily				

* "Monthly" indicates the monthly transfers for culture and "Daily" indicates a 24/48 hr serial transfer (added to control number)
NR = None Required
TC = Test Culture, applied after daily transfer number

ORGANISM CULTURE TRACKING FORM FOR *Mycobacterium bovis* (BCG)

OPP Microbiology Laboratory

Organism:		MR Number:	

Date	Time	Init.	Subculture Source	Transfer		Media Inoculated (and # inoc.)	Media Prep No.	Incubation Conditions	Comments
				Month*	Week**				

* Indicates the month of the year the culture was transferred; added to MR number
** Indicates the week of the month for the transfer (week 1-5); added to MR number
*** Weekly and TC cultures may be used on days 21-25
TC = Test Culture, applied after weekly transfer number
NR = None Required; NA = Not Applicable; SC = Stock Culture; QC = Quality Control; d = days

TEST MICROBE CONFIRMATION SHEET (Quality Control)

OPP Microbiology Laboratory

Organism:		MRME*** Number:	

Source: Tube/Plate ID	Date/ Initials	Staining Results*	Media Information			Results		
			Name	Prep. No.	Inc. Time/ Temp.	Date/Initials	Colony Characteristics	API/Vitek Log #**

* Record Gram stain results or Acid Fast staining results, GPC = Gram Positive Cocci; GNR = Gram Negative Rods; AFR = Acid Fast Rods

** API profile number

*** MRME notation will be for all organisms except *M. bovis*. Use only MR notation for *M. bovis* (BCG)